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Controlling Damping - Off Disease on Cotton Seedlings Caused by *Rhizoctonia solani* and *Fusarium oxysporum* Via Plant Growth-Promoting Rhizobacteria (PGPR)

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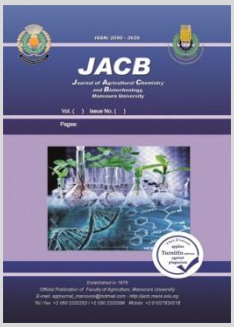

ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that can be enhance the growth of plant directly or indirectly. These bacteria are commonly found in soil associated with roots of plants. Two strains as: *Bradyrhizobium japonicum* PP236808 and *Bacillus subtilis* PP250150 have recorded to increase soyabean plant growth directly. In this study, cotton plays an important role in crop rotation with soybean and maize. So that, aim of this study was to enhance the growth of cotton indirectly. The indirect mechanisms involve the biological control for plant pathogens. *In vitro*, both bacterial strains exhibited antagonistic *Fusarium oxysporum* and *Rhizoctonia solani* causes the cotton damping-off disease through the production of lytic enzymes, IAA, hydrogen cyanide, catalase, ammonia and siderophore. Both strains were positive for phosphate solubilization , IAA production, HCN production and also, were found to be catalase positive. While, *Bradyrhizobium japonicum* PP236808 was highly ammonium. The competition for nutrients leds to improve significantly plant health and promote growth of cotton and consequently seedling survival. Untreated seeds were involved as a control. In greenhouse experiment the treatment of the antagonistic strains (PGPR) significantly repressed the disease incidence to the lowest values as compared to the untreated ones. Also, under field conditions the same PGPR strains significantly decreased the disease incidence. Finally, the application of *Bradyrhizobium japonicum* PP236808 and *Bacillus subtilis* PP250150 as bio-controllar significantly increased seed cotton yield. Since, PGPR are environmentally friendly it is safe to be applied for improving growth of plants and increase yield of crops.

Keywords: PGPR, soil borne fungi, biological control, damping-off, cotton

INTRODUCTION

Cotton (*Gossypium barbadense* L.) is an important crop in Egypt, especially it has an excellent reputation all over the world (Blasingame and Patel 2013). In crop rotation with soybean and maize cotton plays an important role (Hillocks, 2010). In Egypt, cotton seedling damping-off is a disease caused by *Rhizoctonia solani* and *Fusarium* spp. The mentioned pathogens are most commonly involved in the disease (El-Samawaty *et al*. 1999). Biotechnology has opened up new possibilities concerning the evaluation of plant growth by application of beneficial bacteria to the soil and as the biological control of soil-borne pathogens. Plant-growth promoting rhizobacteria (PGPR), are very useful as biofertilizers (Wu *et al*. 2012). These rhizobacteria (PGPR) are able to change physiological parameters of plants by promote growth through several mechanisms such as nitrogen fixation, biological control, production of growth regulators, induction of systemic resistance to pathogens and enhancement of mineral nutrients and water uptake (Conrath *et al*. 2006 & Ryan *et al*. 2008). Apart from rhizobia symbionts, the rhizosphere-associated beneficial bacteria consist of the several bacterial genera, which antagonize phytopathogenic fungi as biological control and these bacteria that develope plant growth promotion (Bashan and Levanony 1990). In addition, these strains of rhizobacteria able to develop growth of plants by producing phytohormones such as indole-3-acetic acid (IAA) (Santoyo *et al.* 2016). Phytohormones which produced by rhizobacteria increase growth of roots by increasing lateral and adventitious roots, which enhance root exudation and available nutrients and water (Spaepen and Vanderleyden 2011). These bacteria automatically colonize the plant roots and antagonize pathogens which widely researched due to the healthy plants (Rajendran and Samiyappan 2008) by producution of some types of antimicrobial materials such as lytic enzymes. The enzymes, cellulase and chitinase are very important to degrade main structure of fungus cell wall by the biological degradation, which cause the fungal mycelia physiologically abnormal and reduce of their infection ability and virulence which is one of the mechanism (Kim *et al.* 2001). From ANOVA tests Aly *et al.* (2022) reported that several species of bacteria exhibited highly significant ability to suppress damping-off disease. Furthermore, different strains of bacteria significantly developed and increased yield of cotton (Aly *et al.* 2021).

The objective of this study was to investigate the antagonistic activities of PGPR strains especially (*Bradyrhizobium japonicum* PP236808 and *Bacillus subtilis* PP250150) against soil-borne fungi (*Fusarium oxysporum* and *Rhizoctonia solani*) to be used as biocontroling agent against damping-off disease on cotton seedlings.

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MATERIALS AND METHODS

Source of bacterial strains

In the Microbiology Department, Faculty of Agriculture, Mansoura University, Mansoura, Egypt, *Bradyrhizobium japonicum* PP236808 was isolated from nodules of soybean plant according to Chhetri *et al*. (2019) and *Bacillus subtilis* PP250150 was isolated by pour-plate method from rhizosphere of soybean plant. Both bacterial strains were identified and screened for production of IAA, GA, proline, EPS and P-solubilization (Nader *et al*. 2024). These activities of the two bacterial strains are presented in Table (1).

Table 1. The activities of *Bradyrhizobium japonicum* PP236808 and *Bacillus subtilis* PP250150 as plant growth promoting strains.

PGPR strains	IAA (mg/100 ml)	GA (mg/100 ml)	Proline (mg/100 ml)	EPS (mg/L)	Phosphate solubilization (mg/100ml)
Bradyrhizobium japonicum PP236808	18.66±0.13a	194.46±0.41c	55.07±0.29c	86.23±0.22a	47.4±1.11a
Bacillus subtilis PP250150	14.44±0.07a	188.09±8.04b	74.5±0.13a	60.35±0.06a	68.92±0.37e

IAA, Indole acetic acid; GA, Gibberellic acid; EPS, Exopolysaccharides.

Data are means ± SD (n=3); different letters within the same group indicate significant differences between means according to Duncan's multiple-range test at $P \leq 0.05$.

Pathogenic fungi

Fusarium oxysporum and *Rhizoctonia solani* isolates were isolated from diseased plants and identified by Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt .

Plant used

Cotton seeds (*Gossypium barbadense* L.) cv. (cultivar Giza 89) were obtained from the Agricultural Research Center (ARC), Giza, Egypt.

Antagonism

In vitro study, the antagonistic activities of both PGPR strains towards damping – off fungi (*F. oxysorum* and *R. solani*) were carried out using plate assay. The inhibition of fungal growth was detected, after the plates were incubated at 28-30ºC for 7 days. After incubation of the plates the inhibition zones were recorded when the control plate was full growth by tested fungus (Sivamani and Gnanamanickam 1988).

Antagonistic materials:

1- HCN production

Qualitative method was used for evaluation of HCN production Kermer and Souissi (2001).

2- Ammonia production

Ammonia production was evaluated by Nessler's reagent according to the method of Dye (1962).

3- Siderophore production

Siderophore production was determined as described by Sharma, *et al*. (2013).

4- Indole Acetic Acid (IAA) production

The IAA content was assayed according to Salkovski's method (Pandya *et al.* 2018).

5- Catalase test

The production of catalase was detected by flooding of 9-10 % solution of hydrogen peroxide to the bacterial biomass on 24 hr old slopes; the evolution of gas bubbles from the growth denoted the presence of catalase (Skerman, 1967).

6- Hydrolytic enzymes production

Hydrolytic enzymes detected by streaking antagonistic PGPR strains individually on the medium containing enzyme substrate (Basha and Ulaganathan 2002). The enzymes were determination according to Ngarajkumer *et al.* (2004). All treatments were carried out in triplicates.

Experimental conditions:

1- Treatment of cotton seed with the antagonistic bacteria

Cotton seeds were surface sterilized by gentle agitation for 3 min. in 2.5% calcium hypochlorite solution. After thorough washing in six changes of sterile distilled water, the seeds were aseptically air dried, placed in flasks containing 150 ml bacterial suspension (10^9 cfu/ml) for 24 hr and sown in greenhouse potted soil and/or field experiment (Mew and Rosales 1986).

2- Preparation of fungal inocula

Substrate for growth of each fungus was prepared in 500 ml glass bottles, each bottle contained 100 g of sorghum grains and 80 ml of water. After 3 weeks when fungal inoclum allowed the fungus-sorghum mixture was air-dried to a powder in a blender and was stored at 5ºC until use.

In the present study, inoculum of each fungus at the rates of 50g /kg soil of *F. oxysporum* and *R. solani* respectively, were infested separately to soil placed on greenhouse. In 20 cm diameter clay pots infested soils were planted with 10 cotton seeds per pot (cultivar Giza 89). Soil without fungi was involved as a control.

Variable of the tested plants

After one week of soils infection, soils were planted with the antagonistic coated seeds and kept under the decided conditions for 5-6 weeks before recording the percentage of diseased seedlings with *F. oxysporum* and *R. solani*. Each treatment of each tested bio-controller was replicated 5 times among twice attempts in the experiments. Finally the produced cotton yield (kentar/fed) was recorded at the end of growth season (Henis *et al.* 1978).

Statistical analysis

Data of greenhouse and field experiments were transformed into arc sine angles before carrying out analysis of variance (ANOVA) to produce approximately constant variance. Management and Analysis of Agronomic Research Experiments (MATAT- C, Michigan State Univ., USA).

RESULTS AND DISCUSSION

The antagonistic effects of PGPR strains

The antagonistic activities of PGPR strains (*Bradyrhizobium japonicum* PP236808 and *Bacillus subtilis* PP250150) against pathogenic fungi (*F. oxysporum* and *R. solani*) were recorded in Table (2) represent the details of the sharp ineffective in inhibiting both pathogenic fungal isolates by the PGPR strains used.

In addition to symbiotic rhizobia, the rhizosphere soil contains beneficial bacteria belong to different genera such as Pseudomonas and Bacillus, which antagonize pathogenic fungi and enhance plant growth (Bashan and Levanony 1990). Several microorganisms are able to antagonize different *Fusarium* species (Sundaramoorthy and Balabaska, 2013). Also, strains of *B. subtilis* exhibited

highly inhibitory activites against *Fusarium* species *in vivo* (Abdelmoteleb, *et al.*, 2017). In addition, rhizobacteria reduce phytopathogenic fungi by different mechanisms and cause suppression of these fungal pathogen (Sofy *et al.* 2021 & Ashour and Afify 2024).

Table 2. Inhibition zones (mm) of mycelial growth of both fungal isolates by PGPR strains

Treatment	Inhibition zone (mm)		Means (mm)
	F. oxysporum	*R. solani*	
Bradyrhizobium japonicum PP236808	11	11.5	11.25
Bacillus subtilis PP250150	13	12	12.5
Control (only fungus)	0.0	0.0	0.0

0.0 = full growth of fungus = no inhibition zone

Detection of antagonistic substances produced by PGPR strains

Results from PGPR activities as antagonistic substances are presented in Table (3). Both strains of bacteria produced HCN, when the deep brown color of filter paper by *B. subtilis* was observed this an indication of HCN production. While, *Bradyrhizobium japonicum* produced copious ammounts of ammonia in pepton water. Appearance of pink color with addition of Salkowski's reagent indicated that IAA was produced by both bacterial strains. For siderophore production *Bacillus subtilis* showed a strong positive reaction (big orange color zone) (Saha *et al.* 2016). Bacterial strains may protect plants from phytopathogens by production of some materials such as ammonia, hydrogen cyanide and catalase (Kremer and Souissi 2001 & Khan *et al*. 2018). The production of plant growth regulators like IAA improve plant growth (Hameeda *et al.* 2008 & Afify and Ashour 2018). The hydrolytic enzymes that have antagonistic activities produced by the two strains tested (*Bradyrhizobium japonicum* PP236808 and *Bacillus subtilis* PP250150) are presented in Table (3). Strain *B. subtilis* recorded the highest chitinase, followed moderate chitinase by *Bradyrhizobium japonicum*. But, both strains produced catalase and not produced cellulase. Jaganmohan *et al.* (2010) demonstrated that some hydrolytic bacteria are very important agents for the protection many plants from phytopathogenic fungi. Thus, hydrolytic soil bacteria that are able to lyse hyphal fungi because fungi are an important source of substrates for hydrolytic enzymes that produced by soil bacteria. Amrih and Elisa (2017) demonstrated that many reports introduced *Bacillus subtilis* with production of lytic enzymes, such as chitinase, protease, lipase, for inhibition various soil borne plant pathogens. The study suggests that several mechanisms may be employed in the inhibition of *F. oxysporum* and *R. solani* by *Bacillus* spp. (Guetsky *et al.* 2002) and with antagonistic bacterial species. In the same Table a study by Kumar *et al.* (2022) showed that several genera of bacteria produce chitinase enzyme as the antifungal mechanism against plant pathogenic fungi by degrading chitin the fungal cell wall component. This mechanism by chithnase enzyme which can produced to inhibit and control pthogenic fungus growth ((Khan *et al.* 2018 & Khairah *et al.*, 2023). In addition, the bioagents materials showed that all bacterial strains produce ammonia and catalase enzyme (Afify and Ashour 2024).

Table 3. Detection of antagonistic substances by PGPR strains

PGPR strains	Produced - substances							
	HCN	NH_3	Siderophore	IAA	Chitinase	Cellulase	catalase	phosphatase
Bradyrhizobium japonicum PP236808	++	+++	+	+	++	-	+	+
Bacillus subtilis PP250150	+++	+	+++	+	+++	-	+	+

Indicator of production:
+++ = high production ; ++ = moderate production ; + = few production ; - = not production

In greenhouse experiment data in Table (4) indicated that, antagonistic bacteria significantly decreased the disease incidence of two pathogenic fungi as compared with the untreated (None). Among the biocontrol agents, *B. subtilis* reduced the disease incidence to the lowest value, *i.e*., 40.00% while untreated treatment exhibited the maximum value viz, 60.00%. Each strains from PGPR were found to gave good disease controlling comparing with untreated treatment (Table 4). These data are in agreement with the eariler reports (Xu *et al.* 1993) and also, with recantly reports Henrique, *et al.* (2020) who concluded that the bacterial strains such as: *B. velezensis*, *B. amyliquefaciens* and *Paenibacillus* sp. inhibited *Colletotrichum gossypii* var. *cephalosporioides* (CGC) in both conditions greenhouse and field experiments. By ANOVA, Aly *et al.* (2022) recorded that strain of *Bacillus* showed highly significant activity in suppressing damping off disease.

Table 4. The effects of PGPR as biocontrol agents on the incidence of cotton seedling damping off under greenhouse condition.

Biocontrol agents (PGPR strains)	Diseased-Seedlings%[b] Pathogenic fungi			Mean
	None	*F. oxysporum*	*R. solani*	
None	97.0 (81.00)	60.0 (50.82)	60.0 (50.82)	72.33 (60.88)
Bradyrhizobium japonicum PP236808	55.0 (47.89)	50.0 (45.00)	50.0 (45.00)	51.66 (46.00)
Bacillus subtilis PP250150	65.0 (53.94)	62.5 (52.28)	40.0 (39.17)	55.33 (47.90)
Mean	72.33 (58.00)	57.5 (49.33)	50.0 (45.00)	

b Percentage data were tranformed into arc-sine angles .
LSD for biocontrl agents (3.294 P< 0.05)

Data in Table (5) indicated that the biocontrol agents as a whole significantly increased the seedlings survival as compared to the untreated control (None) which gave 31.25%. Although *Bradyrhizobium japonicum* PP236808 and *Bacillus subtilis* PP250150 were effective in increasing the seedlings survival in cotton giving the values 73.75% and 70.83% respectively, comparing with 31.25% for untreated treatment. Hence, these data are in full agreement with those recorded by Kaur and Mukhopadhyay (1992). In addition, Henrique, *et al.* (2020) recorded that some strains of bacteria are important tools to increase high cotton yield and fiber of good quality.

Table 5. The effect of PGPR as biocontrol agents on the incidence of cotton seedlings disease under field conditions

Biocontrol agents (PGPR strains)	Seedlings survival %[a] Pathogenic fungi None	*F. oxysporum*	*R. solani*	Mean
None	33.00 (35.05)	28.25 (32.07)	32.50 (34.75)	31.25 (33.21)
Bradyrhizobium japonicum PP236808	82.50 (65.47)	69.00 (56.19)	69.75 (56.70)	73.75 (63.80)
Bacillus subtilis PP250150	77.50 (61.78)	67.75 (55.43)	67.25 (55.14)	70.83 (57.5)
Mean	64.33 (54.10)	55.00 (47.89)	56.5 (48.86)	

LSD for biocontrl agents (2.26 P< 0.05) **[a] Percentage data were tranformed into arc-sine angles.**

Among the biocontrol agents *Bradyrhizobium japonicum* PP236808 increased seed cotton yield significantly up to the highest value *i.e.,* 4.74 kentar/fed (Table 6). The similar application of bacterial strains for reducing cotton seedlings damping-off under field conditions, as reported here is in agreement with Aly *et al.* (2021) who reported that different strains of bacteria significantly developed and increased yield of cotton.

Table 6. Effect of PGPR as biocontrol agents on seed cotton yield (kentar*/feddan) under field conditions

Biocontrol agents (PGPR strains)	Seed cotton yield (kentar*/feddan) None	*F. oxysporum*	*R. solani*	Mean
None	3.65	3.23	3.34	3.40
Bradyrhizobium japonicum PP236808	4.20	4.97	5.06	4.74
Bacillus subtilis PP250150	3.83	3.99	4.51	4.11
Mean	3.89	4.06	4.30	

LSD for biocontrol agents (0.4556 P< 0.05) ; *Kentar = 157.5 kg

CONCLUSION

The strains of plant growth promoting rhizobacteria (PGPR) controlled damping-off fungi on cotton plants in both conditions greenhouse and field experiments. Therefore, the bacterial strains *Bradyrhizobium japonicum* PP236808 and *Bacillus subtilis* PP250150 produced the best results in reducing the incidence of cotton seedlings disease and cotton yield, performing even better than the untreated cotton plants. These strains from PGPR could be a best tool as a bioagents for phytopathogenic fungi and could be used for evaluation in crop rotation with soybean, maize and cotton.

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مقاومة مسببات مرض موت البادرات فى القطن الريزوكتونيا سولانى و الفيوزاريوم أوكسيسبورم بواسطة الريزوبكتيريا المشجعة لنمو النبات

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الملخص

توجد البكتيريا المشجعة لنمو النبات فى ريزوسفير تربة النباتات وهى تعمل بطريقة مباشرة أو غير مباشرة على تحسين نمو النبات وبالتالى زيادة المحصول. من هذه البكتريا سلالتان أظهرتا تحسين وبالتالى زيادة فى محصول فول الصويا هذه السلالات هى: *Bradyrhizobium japonicum* PP236808 and *Bacillus subtilis* PP250150 وبما أن للدورة الزراعية أهمية كبيرة عند زراعة المحاصيل وخصوصا دورة زراعة فول الصويا والقطن والذرة فقد أتبع ذلك فى هذه الدراسة إضافة السلالتان من البكتيريا المذكورة لتحسين نمو وكذلك زيادة محصول نبات القطن بالطريقة الغير مباشرة للبكتيريا المشجعة لنمو النبات كعوامل للمقاومة الحيوية للمسببات المرضية الفطرية مثل فطريات أمراض موت البادرات حيث تضم الدراسة هنا إثنان من العزلات الفطرية *Fusarium oxysporum* and *Rhizoctonia solani* . وقد أوضحت نتائج الدراسة مايلى: سجلت سلالتان البكتيريا تحت ظروف المعمل قدره عالية فى تثبيط نمو فطرى الفيوزاريوم أوكسيسبورم والريزوكتونيا سولانى وذلك من خلال إختبار التضاد عند تنمية هذه البكتريا مع الفطريات الممرضة المختارة بالمقارنة بالنمو الفطرى بمفرده فى نفس ظروف التنمية من البيئة الغذائية ودرجة حرارة النمو للبكتيريا والفطريات. بالإضافة إلى ذلك فقد وجد أن لهذه البكتريا القدرة على إنتاج بعض المواد المضادة لنمو الفطريات الممرضة, حيث سجلت قدرتها على إنتاج الإنزيمات المحلله لجدر هذه الفطريات مثل السليلوليز والكيتينيز. كذلك وجد أن هذه البكتيريا تنتج مواد سامة لهذه الفطريات مثل سيانيد الهيدروجين والأمونيا ومواد أخرى تؤثر على نمو ونشاط هذه الفطريات مثل السايدروفورز وإنزيم الكتاليز. أما عند إختبار هذه السلالات البكتيرية فى تجارب زراعة القطن تحت ظروف الصوبة فى تربة ملوثة بعزلات الفطرين سجلت سلالات البكتيريا الإثنان فعاليتها فى زيادة النسبة المئوية للبادرات الباقية على قيد الحياة. كما أدى إستعمال سلالات البكتيريا تحت ظروف الحقل فى موسم زراعة القطن إلى زيادة مئوية فى نسبة الإنبات وأيضا فى محصول القطن الزهر. من هذه الدراسة نجد أن تعتبر البكتيريا المشجعة لنمو النبات آمنه وصديقة للبيئة وتعمل على تحسين نمو النباتات وزيادة المحصول بالإضافه إلى حماية النبات من الإصابة بالأمراض.